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Transmission of Black Creek Canal Virus Between Cotton Rats

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Black Creek Canal (BCC) virus is a hantavirus associated with hantavirus pulmonary syndrome in southeastern North America. The virus was isolated from the spleen of a cotton rat (*Sigmodon hispidus*) trapped in southern Florida. Our previous studies have shown that we could consistently infect male cotton rats with BCC virus in the laboratory. These animals became persistently infected and virus could be detected in salivary glands, urine, and feces. In this report we show: (1) female and male cotton rats are equally susceptible to BCC virus infection, (2) susceptibility to infection was not influenced by age, (3) all inoculated rats transmitted the infection to uninoculated cage mates, and (4) offspring of infected rats became infected despite the presence of high maternal antibodies. The course of BCC virus infection, as determined by antibody response and the ability to isolate or detect virus, appeared to be similar regardless of whether the rats obtained their infection by inoculation or contact with inoculated rats. ***J. Med. Virol. 60:70–76, 2000.***

KEY WORDS: hantavirus; rodent; reservoir

INTRODUCTION

Hantavirus pulmonary syndrome (HPS) is a severe respiratory disease with a high mortality. The signs and symptoms of HPS include an abrupt onset of fever, headache, and myalgia, followed by the rapid development of respiratory failure. Since the identification of Sin Nombre (SN) virus as the major etiologic agent of HPS in North America, other related hantaviruses have been identified as etiologic agents of HPS. Black Creek Canal (BCC) [Ravkov et al., 1995], Bayou (BAY) [Morzunov et al., 1995], and New York (NY) [Hjelle et al., 1995] viruses are associated with HPS occurring in North America. HPS has also been identified in some South American countries, including Paraguay [Williams et al., 1997], Argentina [Lopez et al., 1996; Levis et al., 1997; Wells et al., 1997], Chile [Centers for Disease Control, 1997], and Brazil [Da Silva et al., 1997; Vasconcelos et al., 1997].

Hantaviruses are maintained in nature in rodent reservoirs and each virus is associated primarily with a specific rodent species. Experimental studies with Hantaan (HTN) [Lee et al., 1981a, 1981b], Puumala (PUU) [Gavrilovskaya et al., 1990], Seoul (SEO) [Morita et al., 1985], and BCC [Hutchinson et al., 1998] viruses suggest that although there are many similarities in the pathogenesis of hantaviruses in reservoir rodents, the dynamics of infection and transmission of virus may differ between the various hantavirus/reservoir pairs. Previously we reported that male cotton rats became persistently infected after a subcutaneous injection of 1,000 tissue culture infectious dose (TCID) [Hutchinson et al., 1998]. The study presented here was designed to determine if: (1) inoculated animals could transmit virus to naive animals, (2) females were also susceptible, (3) age influenced infection, and (4) vertical transmission had occurred.

MATERIALS AND METHODS

Virus

The BCC virus used in this study was originally isolated from the lung of an infected cotton rat (*S. hispidus*) trapped near the residence of an HPS case in southern Florida [Rollin et al., 1995]. After its initial isolation, the virus was passaged three times in Vero E6 (E6) cells in an attempt to increase the viral titer. Infectious viral titers were obtained by infecting E6 cells with $\log_{10}$ dilutions of virus and using an indirect fluorescent antibody (IFA) procedure to determine the TCID.

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Animal Inoculations and Sample Collection

Cotton rats were obtained from Virion Systems Inc. (Rockville, MD) and were transferred to the biosafety level 4 (BSL 4) laboratory. Rats ranging in age from 3 weeks to 4 months were anesthetized individually with metofane, weighed, pre-bled from the retro-orbital plexus, and inoculated subcutaneously with 0.5 ml sterile saline (sham-inoculated) or 0.5 ml sterile saline containing 1,000 TCID of BCC virus (BCC inoculated). BCC-inoculated rats were paired with sham-inoculated rats of the same age and size, immediately following inoculation. Cages were covered with polyester filter bonnets and placed inside a Laminar Flow isolator (Lab Products, Maywood, NJ). All animals were bled periodically throughout the 12 weeks of the study. At 12 weeks postinoculation (p.i.), rats were bled by cardiac puncture and killed. In addition, tissue samples (salivary gland, lung, liver, spleen, and kidney) were taken, and individually frozen at −70°C, from all occupants of cages that contained either a pregnant female or suckling pups born within the 12th week. Half of each litter born during the final week of the study were bled and killed during the first 48 hr after birth and the remaining half at 1 week after birth. Tissue samples were tested for infectious virus, viral complementary RNA (vcRNA), and viral antigen (pups and fetuses only). The entire midsection (between the shoulders and hips) of fetuses was homogenized as one sample, and used to detect infectious virus and vcRNA.

Virus Isolation

The frozen tissues were thawed once, homogenized in an equal volume of minimal essential medium containing Earle's salts, 2% fetal bovine serum (FBS), 0.29 mg/ml L-glutamine, 0.2 mg/ml gentamycin, and amphotericin B (MEM-M). Homogenates were diluted to 10% in MEM-M. Two hundred microliters were placed in Eppendorf tubes (Marsh Scientific, Rochester, NY) containing 500 μl guanidinium-isothiocyanate for RNA purification (described below) and 200 μl were used for identification of infectious virus. Three hundred microliters of MEM-M were added to the 200 μl of 10% tissue homogenate and this was inoculated onto E6 monolayers in 25-cm^2 plastic tissue culture flasks. Virus was allowed to adsorb to the cells for 1 hr, followed by the addition of 5.0 ml of MEM-M. Cells were incubated at 37°C in 5% CO_2. Medium was changed on day 7. On day 14, 0.2 ml of cell supernatant was removed from the tissue culture flasks and used to infect cell monolayers on 16-well chamber slides (Nunc Inc., Naperville, IL). The medium was changed on days 3 and 9. On day 14, the cell monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.2, fixed in cold acetone, air-dried, gamma irradiated (1×10^6 rads), and tested for the presence of BCC antigen by an IFA test, using anti-BCC virus primary antibody, which had been prepared by inoculation of adult cotton rats with purified BCC virus. The sera did not react with uninfected E6 cells or with samples from uninoculated rats. A goat anti-*Peromyscus leucopus* (also a sigmodontine rodent) IgG fluorescein isothiocyanate conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as the secondary antibody.

Purification and Detection of vcRNA

RNA was purified using the acid-guanidinium isothiocyanate-phenol chloroform method as previously described (Chomczynsky and Sacchi, 1987). The cDNA synthesis step was carried out using a genomic sense primer specific for nucleocapsid vcRNA (mRNA plus antigenomic RNA). Because vcRNA is not packaged into mature virions (data not shown) we were confident that this method was accurate and sensitive for identifying the sites of viral activity. The samples were amplified for 40 rounds using a primer set described previously for the closely related hantavirus SN [Hutchinson et al., 1996]. After amplification, the PCR product was detected by hybridization to an electrochemiluminescent [tris(2,2′-bipyridine)-ruthenium(II) chelate] (TBR)-labeled probe (5′-[TBR]-AAA CCT GTT GAT CCA ACA GGG ATT GAI C-3′). The hybridized product was captured on streptavidin-coated magnetic beads. The bead-bound product was collected by an external magnet, washed to remove any unhybridized TBR-labeled probe, and stimulated by an electrochemical reaction to a high-energy state. Light, released upon relaxation to the ground state, was detected using a photomultiplier tube and converted to a digital output. Each RNA sample was run in triplicate and considered positive if its signal was greater than five times the background signal.

Serology

Blood collected throughout this experiment were screened for the presence of IgG against BCC virus by an IFA test and by enzyme-linked immunosorbent assay (ELISA). In the IFA procedure, confluent monolayers of E6 cells grown on T75 tissue culture flasks were infected with BCC virus. Cells were harvested by scraping the monolayers 14 days p.i., cells were washed with sterile PBS, pH 7.2, resuspended in 90% FBS, aliquoted, and gamma irradiated with 5×10^6 rads. Irradiated cells were stored at −70°C. Cells were thawed, washed with PBS, pH 7.2, and spotted onto Teflon-coated, 12-well slides. The slides were air-dried and fixed in cold acetone. Bloods were diluted fourfold (1:25–1:6400) in PBS, pH 7.2 and placed in individual wells of the slides. Slides were incubated for 1 hr at 37°C in a humidified chamber and then washed twice for 10 min each with PBS, pH 7.2. Fluorescein isothiocyanate-conjugated goat anti-*Peromyscus leucopus* IgG was added to the slides, which were then incubated for an additional hour as above. Slides were washed, mounted in 10% glycerol, and examined using a Axioskop microscope (Zeiss, Oberkochen, Germany). Samples were considered positive if 30% or more of the cells were fluorescent. Negative controls included bloods from uninoculated animals as well as uninfected E6 cells incubated with a known positive blood.

The ELISA for viral IgG was performed as described previously [Ksiazek et al., 1995]. Briefly, polyvinyl chloride microtiter plates were coated overnight at 4°C with antigen (Ag) derived from a basic buffer-detergent extract of E6 cells infected with BCC virus (BCC Ag) or uninfected E6 cells (E6 Ag), both of which had been subjected to gamma irradiation (5×10^6 rads). Whole blood samples were serially diluted in 5% skim milk in PBS-Tween and allowed to react with the Ag-coated wells. A mixture of goat anti-*Rattus* and goat anti-*Peromyscus* IgG conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used to detect bound IgG. Optical densities (ODs) were measured at 410 nm, and adjusted OD_{410} values were obtained by using the equation: OD of BCC-Ag-coated wells minus OD of E6-Ag-coated wells.

Detection of Viral Antigen in Tissues

Immunohistochemistry (IHC) assays were performed using a labeled streptavidin-biotin method described previously for the detection of SN virus antigen [Zaki et al., 1995]. Briefly, 4-μm sections of paraffin-embedded tissues were placed on Fisher Plus slides (Fisher Scientific, Pittsburgh, PA), deparaffinized, and rehydrated in graded alcohol incubations. The tissue sections were digested in 0.1 mg/ml Proteinase K (Boehringer Mannheim Corporation, Indianapolis, IN) dissolved in 0.6 M Tris HCl, pH 7.5/0.1% $CaCl_2$ solution. A blocking step was performed using 20% normal swine serum in Tris-saline-triton (NSS/TST) buffer solution. The primary antibody, a polyclonal antibody prepared by inoculating rabbits with purified BCC virus in complete Freund's adjuvant, was applied to the tissue sections and incubated at room temperature for 90 min followed by a 15-min incubation at room temperature with biotinylated swine anti-rabbit antibody and a subsequent 15-min incubation at room temperature with streptavidin alkaline phosphatase conjugate (LSAB kit, Dako Corporation, Carpinteria, CA). The alkaline phosphatase activity was detected using naphthol/fast red substrate (Dako Corporation) and the sections were counter stained in Mayer's hematoxylin (Fisher Scientific) and mounted with aqueous mounting medium (Signet Laboratories, Dedham, MA).

Biosafety

All animal work and subsequent work with infectious virus was carried out inside a BSL 4 laboratory. Blood samples and chamber slides were double bagged in heat-sealed bags and passed to the outside through a tank of 3% Lysol (Reckitt and Colman, Inc., Montvale, NJ). Before being opened, the bags were placed in a container of dry ice and treated with gamma irradiation (5×10^6 or 1×10^6, respectively).

RESULTS

The first two questions we wished to address were whether the age and/or sex of the rats appeared to influence their susceptibility to BCC virus infection. All BCC-virus-inoculated rats had circulating antibodies specific for BCC virus by 14 days p.i. Whereas none of the sham-inoculated animals had seroconverted by day 14, by 35 days p.i. all had BCC-specific antibodies circulating in their blood (Table I). These data show that BCC virus can be transmitted horizontally from infected to naive rats. Furthermore, neither age (in the range tested) nor sex appeared to influence susceptibility to infection nor the ability of inoculated rats to transmit virus to sham-inoculated cage mates.

TABLE I. Horizontal Transmission of BCC Virus From Inoculated to Naive Cotton Rats

Cage #	Animal identification[a,b]	Inoculum	Age[c]	BCC Ab[d]
1	1M	Saline	3	day 35
1	IF	BCC	3	day 14
2	2M	BCC	3	day 14
2	2Fa	Saline	3	day 35
2	2Fb	Saline	3	day 35
3	3M	Saline	3	day 35
3	3F	BCC	3	day 14
4	4M	Saline	6	day 35
4	4F	BCC	6	day 14
5	5Ma	BCC	6	day 14
5	5Mb	Saline	6	day 35
6	6M	Saline	7	day 35
6	6F	BCC	7	day 14
7	7M	Saline	7	day 35
7	7F	BCC	7	day 14
8	8M	BCC	7	day 14
8	8F	Saline	7	day 35
9	9M	BCC	7	day 14
9	9F	Saline	7	day 35
10	10M	BCC	7	day 14
10	10F	Saline	7	day 35
11	11M	Saline	16	day 35
11	11F	BCC	16	day 14
12	12M	BCC	16	day 14
12	12F	Saline	16	day 35

BCC, Black Creek Canal.
[a]M, male; F, female.
[b]In the cages housing more than one rat of the same sex they were distinguished by clipping the left ear of one of them (rat a), or the right ear (rat b).
[c]Age, in weeks, or the rats at the time of inoculation.
[d]The time p.i. when anti-BCC virus-specific antibodies were first detected.

We also wished to determine if the immune response appeared to be similar in the BCC inoculated (artificially infected) and the naive cage mates (naturally infected). As can be seen in Figure 1, the antibody-mediated immune response, although delayed in the sham-inoculated cage mates, is comparable to that seen in the BCC-inoculated animals (Fig. 1).

Twelve weeks p.i. all rats were bled by cardiac puncture and killed. In an attempt to determine if vertical transmission also occurred, tissue samples were taken from all occupants of cages that contained either a pregnant female or pups born within 6 days of being killed. These samples were tested for the presence of infectious virus and vcRNA. All animals tested, including pups, had high levels of circulating anti-BCC antibody. In six of eight adults, either infectious virus or vcRNA was detected. Whereas vcRNA was not detected in any of the fetal samples, five of the six fetuses were

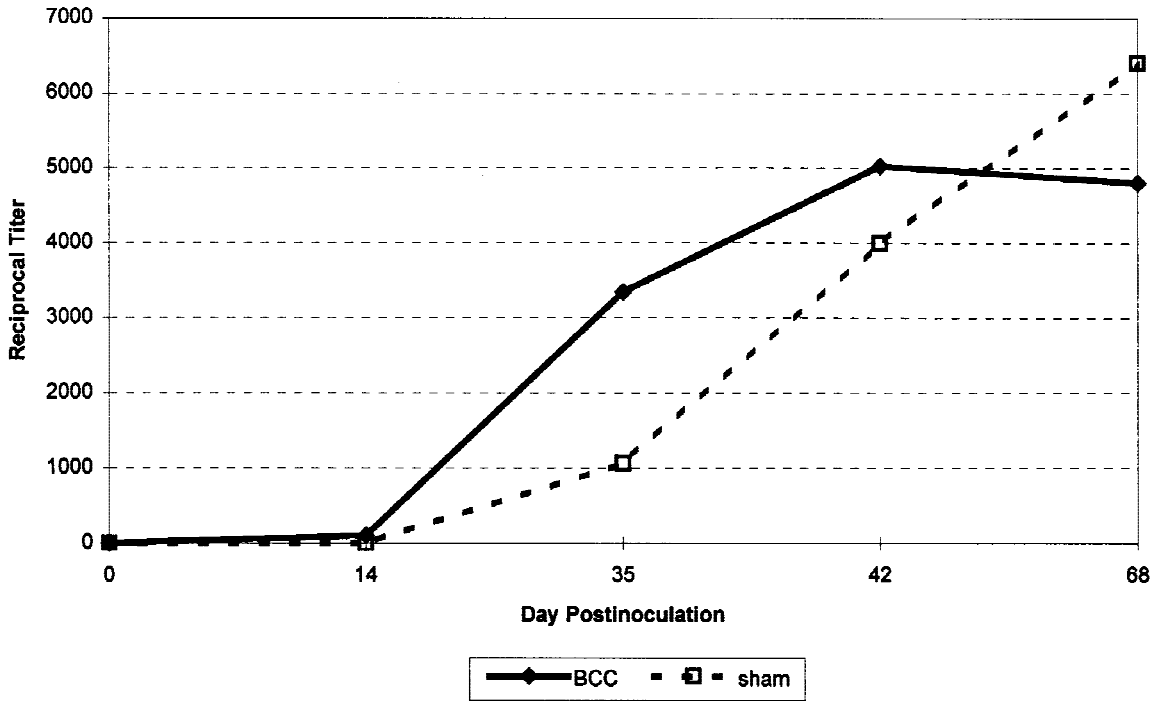

Fig. 1. Comparison of the humoral immune responses against Black Creek Canal (BCC) virus in BCC-injected rats versus those infected through contact. The results are presented as the average antibody titers as measured by enzyme-linked immunosorbent assay (ELISA).

positive for infectious virus. Virus was isolated from one of the five 2-day-old pups and three of the five 6-day-old pups. Unlike the results seen in the fetal samples, vcRNA was detected more frequently than infectious virus in nursing pups. Three of five pups were positive for vcRNA at 2 days of age and could be detected in all (5/5) of the 6-day-old pups (Table II).

In an attempt to determine which cells were infected and the extent of the infection, IHC was used to detect viral antigen. Unfortunately the IHC results were mostly negative; viral antigen could be detected only in the lung of one 6-day-old pup. Even in this pup antigen staining was sparse and confined to endothelial cells and macrophages (Fig. 2).

DISCUSSION

Hantaviruses are maintained in nature in rodent hosts and each virus is associated with a single rodent species. The exact mechanism(s) by which each virus is transmitted within its rodent host community is poorly understood. Serosurveys on wild caught *P. maniculatus* [Childs et al., 1994; Mills et al., 1997], *R. norvegicus* [Childs et al., 1987a, 1987b], and *S. hispidus* [Glass et al., 1998], have shown that there is a direct correlation between increased size and presence of anti-hantavirus antibodies. This finding may indicate that most animals become infected after reaching maturity and/or that animals that are infected early in life are less likely to survive and thus are not captured. Previous laboratory infection studies with SEO virus in *R. norvegicus*, its natural host species, have suggested that newborn rats (less than 48 hr after birth) are more susceptible to hantavirus infection [Morita et al., 1985; Tanishita et al., 1986; Kariwa et al., 1996]. In these studies, SEO virus replicated to higher titers, could be detected in more organs, and resulted in the death of more than 50% of inoculated newborn animals. Infected newborns were also capable of transmitting the virus to naive cage mates. The results from inoculation of adult rats differed greatly from that seen in newborns with little virus replication and no transmission. The conflict between the laboratory results and field serological studies may be explained by the passage history of the virus isolate used. Other studies have shown that infection and transmission of SEO virus differs greatly between isolates and the use of cell culture for isolation and propagation of virus can result in the selection of virus with a reduced infectivity for rodents [Morita et al., 1994].

In nature, serologic evidence of infection is predominantly found in male *P. maniculatus* [Childs et al., 1994; Mills et al., 1997] and *S. hispidus* [Glass et al., 1998]. Whereas this observation is believed to be due to the fact that the males are much more aggressive, it is also possible that susceptibility to hantavirus infection differs between males and females. The data presented in Table I indicate that neither the age (from 3 weeks to 4 months) nor the sex of the rats appeared to influence susceptibility to BCC virus infection. All BCC-inoculated animals became infected, with the appearance of anti-BCC virus-specific antibodies occurring within the first 2 weeks. Additionally, transmission to naive cage mates occurred with equal efficiency regardless of age or sex. It is important to note that not only

TABLE II. Vertical Transmission of BCC Virus

Animal identification	Age[a]	Ab titer	Virus isolation[b]	vcRNA
4M		6400	NEG	NEG
4F		6400	SAL. GL., LUNG, LIV.	SAL. GL., LUNG, LIV., KID.
4FETUS1	Fetus	ND	NEG	NEG
4FETUS2	Fetus	ND	POS.	NEG
4FETUS3	Fetus	ND	POS	NEG
2M		6400	NEG	NEG
2Fa		6400	SAL. GL.	SAL. GL., LUNG, SPL.
2FETUS1	Fetus	ND	POS.	NEG
2FETUS2	Fetus	ND	POS.	NEG
3FETUS3	Fetus	ND	POS.	NEG
8M		6400	SAL. GL., SPL.	SPL., KID.
8F		6400	NEG	LUNG, KID.
8BABY1	2 Days	1600	NEG	NEG
8BABY2	2 Days	1600	NEG	LIV.
8BABY3	6 Days	6400	LUNG, LIV., SPL.	SAL. GL., LUNG, LIV., SPL., KID.
8BABY4	6 Days	6400	NEG	SAL. GL., LUNG, LIV., SPL., KID.
10M		6400	SAL. GL., SPL.	SAL. GL., SPL., KID.
10F		6400	SAL. GL., SPL.	LUNG, KID.
10BABY1	2 Days	6400	NEG	NEG
10BABY2	2 Days	6400	LIV.	KID.
10BABY3	2 Days	6400	NEG	LIV.
10BABY4	6 Days	1600	NEG	LUNG, LIV., KID.
10BABY5	6 Days	6400	LIV., KID.	LIV., KID.
10BABY6	6 Days	6400	LIV., KID.	SPL., KID.

BCC, Black Creek Canal.
[a]The age of the pups at the time of euthanasia.
[b]For both the virus isolation and vcRNA, NEG indicates none of the samples were positive; SAL. GL., salivary gland; LIV., liver; SPL., spleen; KID., kidney.

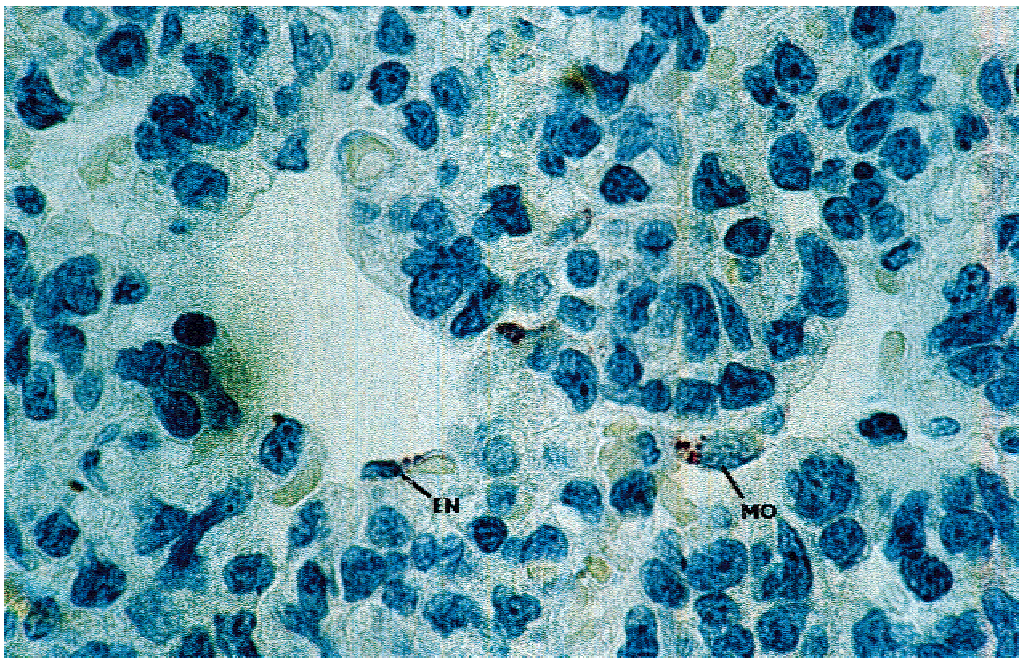

Fig. 2. Presence of Black Creek Canal (BCC) viral antigen in pup born to an infected dam. Photomicrograph (100×) of a lung section from a 6-day-old cotton rat showing rare positive staining in endothelial cells (EN) and macrophages (MO).

was the frequency of transmission 100%, but transmission occurred very rapidly, most likely within the first 11 days of pairing. At the end of the study period the presence of virus did not appear to depend on whether the animals were infected by subcutaneous injection or from infected cage mates, lending further support to our model system being a close representation of natural infection. Thus the most likely explanation for the low percentage of infected females in nature is that transmission takes place primarily through aggressive behavior. In the laboratory, we are dealing with a closed system in which animals are confined to a relatively small area, resulting in closer contact and probably also an increase in aggressive behavior among the

females. In these studies, paired cotton rats were extremely aggressive regardless of sex. Even though animals were closely matched according to age and weight, a few animals were killed by their mates. All adult animals showed some evidence of fighting.

Figure 1 shows a comparison of the anti-BCC virus antibody response in inoculated and naive cage mates. In the naive cage mates, the BCC virus-specific response was delayed as would be expected; however, the antibody response was very similar in virus-inoculated and naive (naturally infected) animals. We are currently developing materials in which to examine the cell-mediated immune response in cotton rats, and perhaps other *Sigmodontine* rodents. We believe these studies will be invaluable in helping to understand how the virus can persist in rodents with no ill effects and cause such devastating disease in humans.

Laboratory infection studies have also shown that maternal antibodies are capable of protecting pups from infection when virus is inoculated subcutaneously [Dohmae et al., 1993]. The data presented in Table II and Figure 2 show that suckling cotton rats can and do become infected with BCC virus even in the presence of high titer anti-BCC antibodies. Conception occurred approximately 30–40 days after the dams became infected. We would expect, based on previous studies [Hutchinson et al., 1998], this time period to be after the virus had been cleared from the circulatory system and during a time when infectious virus titers in peripheral tissues decline rapidly. Whereas maternal antibodies could not prevent vertical transmission, in these studies, they may possibly have reduced the extent of infection because viral replication in suckling animals appears to be very limited. It is as yet unclear if BCC virus can be transmitted in utero. Although infectious virus could be recovered from most fetuses, this finding may represent contamination from the infected dams, which had virus circulating in the bloodstream at the time of euthanasia. The presence of vcRNA (3 of 5) and infectious virus (1 of 5) in 2-day-old pups is suggestive of either in utero or perinatal transmission. Previously we showed that vcRNA and infectious virus had already reached high levels by 7 days p.i., so it is currently unknown how early after inoculation either of these could be detected [Hutchinson et al., 1998]. The ability to isolate/detect virus more frequently in dams may indicate that virus replication increases in females during pregnancy and nursing (Table II). In two cases (2M and 4M) we were unable to detect either infectious virus or vcRNA from the sires. It is possible that these males had cleared their infection but because only a few tissues were tested we cannot rule out a persistent low level infection in these animals.

Viral infection appeared to have no effect on reproduction: infected dams had as many litters of similar size as naive animals; and the size and growth patterns were the same for pups born to infected and naive rats (data not shown). We concluded that both horizontal and vertical transmission of BCC virus occurs among cotton rats housed together. Further studies are planned to examine the mechanisms of transmission in more detail to determine: (1) if direct contact is necessary, (2) frequency of vertical transmission (in utero and perinatally), (3) differences in efficiency of transmission (horizontal and vertical) during the acute and persistent phase of infection, (4) differences in viral pathogenesis between infected pups and adults, and (5) if suckling animals develop immunity to reinfection as adults. BCC virus has been detected only in cotton rats from Southern Florida. The cotton rats used in this study originated from the Midwestern United States; therefore, it would be of great interest to determine if BCC infection of cotton rats obtained from Southern Florida differs from our studies.

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